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Substitution of the Unique Cysteine Residue of Murine Hsp25 Interferes with the Protective Activity of This Stress Protein Through Inhibition of Dimer Formation

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ABSTRACT

Murine small stress protein [heat shock protein 25 (Hsp25)] expression confers thermotolerance and protection against oxidative stress. Hsp25 is an oligomeric ATP-independent phospho-chaperone that can generate a glutathione-dependent pro-reducing state in cells that are normally devoid of small stress protein constitutive expression. Hsp25 contains only one cysteine residue (position 141) that is highly susceptible to oxidation. We have explored the significance of this reactive residue by generating a mutant in which cysteine-141 was substituted by an alanine residue (C141A mutant). We report here that the C141A mutant did not form dimers when expressed in either murine L929 or human HeLa cells, hence, demonstrating that cysteine-141 regulates Hsp25 dimer formation. The C141A mutant also interfered with the dimerization of human Hsp27, a constitutively expressed small stress protein in HeLa cells. The mutated polypeptide showed a decreased ability to multimerize, but its expression was still able to induce cellular protection against oxidative stress. The C141A mutant was, however, less efficient than the wild-type protein in counteracting staurosporine-induced apoptosis, and it showed no *in vivo* chaperone activity. Hence, the cellular protection mediated against different stressors may require specific structural organizations of Hsp25 that are differently altered by the mutation. Of interest, when expressed concomitantly with wild-type Hsp25, the C141A polypeptide induced a dominant-negative effect, a phenomenon that may result from the ability of small stress proteins to interact and multimerize with each other. *Antioxid. Redox Signal.* 7, 436–445.

INTRODUCTION

MAMMALIAN SMALL STRESS PROTEINS [small heat shock proteins (sHsps)] are oligomeric polypeptides related to α-crystallin (17). Expression of several of these proteins was found to confer thermotolerance (22), resistance to cytotoxic drugs (38), or oxidative stress (3, 6, 16, 26, 28, 30, 40, 41, 43, 44, 49–51). Some sHsps can also act as actin capping/decapping enzymes (23, 37). sHsps also counteract apoptosis induced by agents that do not trigger an oxidative stress (1, 2, 5, 42, 45, 47) or that takes place during early differentiation (33, 34). As a consequence, the antiapoptotic activity of human heat shock protein 27 (Hsp27) expression can promote tumorigenesis (14). Hsp27 belongs to the so-called family of "survival proteins" (18), which includes several antiapoptotic proteins (*i.e.,* Hsp70, Bcl-2, IAP, and survivin) whose expression is often up-regulated in cancer cells and results in aggressively growing and therapy-resistant tumors.

Evidence have been obtained that sHsps act as ATP-independent chaperones (15, 20) that bind misfolded polypeptides. This creates reservoirs of folding intermediates (10, 12, 24) counteracting the formation of deleterious protein ag-

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gregates that resist renaturation by ATP-dependent protein chaperones (Hsp70, Hsp40, Hsp90, and cochaperones). This molecular mechanism is probably of prime importance in the cellular protection mediated by sHsps against heat shock. However, in the case of the protection mediated against oxidative stress, the mechanism also involves the modulation of enzymes involved in the reactive oxygen species (ROS)-glutathione pathway, such as glucose-6-phosphate dehydrogenase (G6PDH) and glutathione transferase (30, 44). This modulation upholds glutathione in its reduced state (GSH) and induces a glutathione-dependent decrease in the intracellular level of ROS (30, 44). The protective activity of human Hsp27 and murine Hsp25 in response to oxidative stress correlates with their transient hyperaggregation followed by a phosphorylation-dependent deaggregation of their multimeric forms (28, 32, 43).

Less is known concerning the molecular mechanism generated by sHsps that protects against apoptosis induced independently of oxidative stress. In the case of human Hsp27, this protein appears to act both upstream and downstream of mitochondria by interfering with cytochrome *c* release and cytochrome *c* activation of procaspases (7, 39, 41). Hsp27 also modulates apoptotic regulators such as Daxx (8) and Akt (45).

Human Hsp27 and murine Hsp25 polypeptides, but not αB-crystallin, contain only one cysteine residue whose location in the protein sequence is well conserved (position 141 in murine and 137 in human). This cysteine residue is highly susceptible to oxidation (9, 53, 54) induced by inducers such as molecular oxygen, oxidized glutathione (GSSG), and low-molecular-weight mixed disulfides, nitric oxide, nitrosothiols, peroxynitrite, reactive lipid species, and other electrophilic species. Disulfite formation between two Hsp25 polypeptides has been observed, especially in oxidative stress conditions (9, 53, 54). Oxidative conditions can also promote Hsp25 *S*-thiolation (9).

We report here the effects induced by the substitution of the unique cysteine residue of murine Hsp25 (cysteine-141) by alanine. The mutation impaired Hsp25 ability to form dimers and altered its native size. Expression of the C141A mutant polypeptide in L929 cells still induced a protection against oxidative stress. It was, however, less efficient at counteracting staurosporine-mediated apoptosis and had lost its *in vivo* chaperone activity toward denatured luciferase. Moreover, the C141A polypeptide induced a dominant-negative effect when it was coexpressed with wild-type Hsp25.

MATERIALS AND METHODS

Vectors, cells, and reagents

The cDNA encoding murine Hsp25 was inserted between the *Eco*RI and *Sal*I sites of the mammalian expression vector pCI-neo (Promega, Charbonnieres, France). This gave rise to an expression vector named pCI-neo-Hsp25wt. To generate the single amino-acid substitution C141A, the cysteine codon TGC was replaced with an alanine codon GCC using the Quikchange Site-Directed Mutagenesis kit (Stratagene). The primers used were as follows: sense, GGC TAC ATC TCT CGG **GCC** TTC ACC CGG AAA TAC ACG, and antisense, G CGT GTA TTT CCG GGT GAA **GGC** CCG AGA GAT GTA GCC. The resulting expression vector was named pCI-neo-Hsp25C141A. For firefly luciferase expression, we used the pGL3-Promotor Vector from Promega. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. For transient expression, exponentially growing HeLa or L929 cells were seeded at a density of 1.5 or 2.5 × 10^6 cells/78 cm^2 one night before transfection with 6 or 12 μg, respectively, of vector according to the Lipofectamine™ reagent procedure (Invitrogen, Cergy Pontoise, France). The DNA was left on cells for 3 h; thereafter, cells were washed once with phosphate-buffered saline (PBS) before being further incubated in fresh medium. Forty-eight hours after transfection, cells were submitted to the different treatments. Alternatively, G418 (500 μg/ml) was used to produce a polyclonal population of HeLa cells expressing either Hsp25wt or mutant Hsp25C141A. WT6 cells (L929 cells stably expressing Hsp25wt) have already been described (43). Crystal violet, hydrogen peroxide (H_2O_2), G418, blue dextran, thyroglobulin, apoferritin, alcohol dehydrogenase, and carbonic anhydrase were from Sigma (Sigma–Aldrich, St-Quentin-Fallavier, France). Anti-murine Hsp25 and anti-human Hsp27 antibodies were from Stressgen (Victoria, BC, Canada). Carboxy-2′,7′dichlorodihydrofluorescein diacetate (Carboxy-H2-DCFDA) was from Molecular Probes (Eugene, OR, U.S.A.).

Gel electrophoresis and immunoblotting

After treatment, cells were either immediately scraped or allowed to recover for different time periods at 37°C before being harvested. Cells were rinsed twice in ice-cold PBS and scraped off the dish. At this point, aliquots were withdrawn for protein concentration determination. Thereafter, cells were lysed in boiling sodium dodecyl sulfate (SDS) buffer (62.5 m*M* Tris-HCl, pH 6.8, 1% SDS, 0.1 *M* dithioerythritol, 10% glycerol, and 0.001% bromophenol blue). Alternatively, for detection of Hsp25 dimers, cells were lysed in nonreducing SDS buffer containing no dithioerythritol. One- or two-dimensional immunoblots were performed as previously described (4). The detection of immunoblots was performed with the ECL™ system (Amersham Life Science). Autoradiographs were recorded onto X-Omat LS films (Eastman Kodak Co, Rochester, NY, U.S.A.).

Gel filtration analysis

Forty-eight hours after transfection, cells were washed in ice-cold PBS and scraped from the dishes. Cells were then pelleted at 2,000 *g* for 5 min and lysed by gentle douncing at 4°C in a buffer containing 20 m*M* Tris, pH 7.4, 20 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 0.1% Triton X-100. The lysates were then centrifuged at 20,000 *g* for 10 min and the supernatants applied to a Sepharose 6B gel filtration column (1 cm × 100 cm) (Pharmacia, Uppsala, Sweden) equilibrated and developed in lysis buffer devoid of Triton X-100. The fractions eluting from the column were analyzed in either reducing or nonreducing SDS-polyacrylamide gels. Gels were then processed for immunobloting analysis using antibodies specific for either murine Hsp25 or human Hsp27. Molecular

mass markers used to calibrate the gel filtration column included blue dextran (>2,000,000 Da), thyroglobulin (669,000 Da), apoferritin (440,000 Da), and carbonic anhydrase (29,000 Da).

In vivo *fluorescent measurement of intracellular ROS*

In vivo measurement of intracellular ROS was performed essentially as previously described (27, 30). In brief, 48 h after transfection, L929 cells were incubated for 30 min at 37°C with 5 μg/ml Carboxy-H2-DCFDA and then exposed to oxidative treatments. Cell suspensions were washed with PBS to remove the fluorescent marker. Flow cytometric analysis was performed using a FACS-Calibur cytometer (Becton Dickinson, Le Pont de Claix, France) using 488-nm excitation wavelength. The emission filter was 530-nm bandpass for DCF fluorescence.

Assay for oxidative stress-induced cytotoxicity

Cells (10^4/well) were grown in 96-well plates for 24 h before being analyzed for their resistance to H_2O_2. At different time periods, supernatants were discarded and the remaining viable cells were rinsed twice with PBS buffer and stained for 15 min with 0.5% crystal violet in 50% ethanol. Afterward, plates were rinsed and dried before a medium containing 0.1 *M* sodium citrate, pH 5.4, and 20% methanol was added to solubilize the stained cells. The absorbance of each well was read at 570 nm with an MR5000 microELISA reader (Dynatech Laboratories, Chantilly, VA, U.S.A.). The percentage of cell survival was based on the ratio of the relative absorbance of the different samples to that of untreated cells.

In vivo *Hsp25 chaperone activity*

L929 cells were transiently transfected with DNAs of the firefly luciferase expression pGL3-Promotor vector (Promega) and either the control pCI-neo vector, the pCI-neo-Hsp25wt vector, or the pCI-neo-Hsp27C141A vector. Forty-eight hours after transfection, cells were scraped from the dishes and resuspended in fresh medium. They were then submitted or not to heat shock treatment of either 15 or 20 min at 42°C. Cells were either immediately lysed or allowed to recover at 37°C in a CO_2 incubator for different time periods. Luciferase activity was determined using the Steady-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. In brief, equal volumes of cell suspension and Steady-Glo reagent were mixed, and after incubation at room temperature luminescence was quantified using a Victor Wallach cytofluorometer (EG&G Instruments, Evry, France).

RESULTS

Characterization of the C141A mutation of murine Hsp25

The C141A mutation of murine Hsp25 was analyzed in murine L929 fibrosarcoma that are devoid of constitutive expression of endogenous Hsp25 or αB-crystallin (29). Endogenous Hsp25 expression can only be detected in these cells after a recovery period of at least 12 h after heat shock (29). L929 cells were therefore transiently transfected with either the empty vector pCI-neo, the Hsp25 wild type-bearing vec-

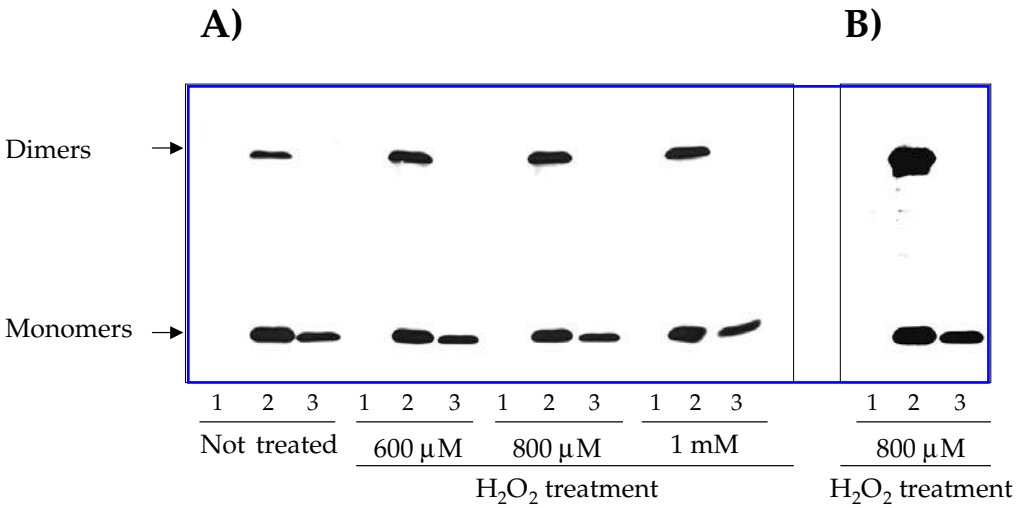

FIG. 1. Characterization of the C141A Hsp25 mutant expressed in murine L929 cells. The experiment was performed in L929 cells devoid of sHsp constitutive expression. **(A)** L929 cells were transiently transfected with either the control pCI-neo vector (lanes 1), the pCI-neo-Hsp25wt (lanes 2), or the pCI-neo-Hsp25C141A (lanes 3) vector. Forty-eight hours after transfection, cells were either left untreated or treated with various concentrations of H_2O_2 to induce Hsp25wt dimerization. Immunoblot analysis of Hsp25 was performed with membranes obtained from SDS-polyacrylamide gels run in nonreducing conditions as described in Materials and Methods. An autoradiograph of an ECL-revealed immunoblot is presented. **(B)** Same as A, but protein loading in the gel was increased (the 800 μ*M* H_2O_2 treatment is presented). Mutant Hsp25 was devoid of dimer formation. Similar observations were made in the case of untreated cells or cells exposed to different concentrations of H_2O_2.

tor pCI-neo-Hsp25wt, or the pCI-neo-Hsp25C141A vector carrying the C141A mutation of Hsp25. Transfection efficiency was estimated to be ~30%. Forty-eight hours after transfection, cells were exposed or not for 1 h to different concentrations of H_2O_2. Total cellular proteins were then separated in nonreducing SDS-polyacrylamide gels (see Materials and Methods), and Hsp25 was detected by immunoblot analysis. Figure 1A shows that, in nontreated cells, a large fraction of wild-type murine Hsp25 (Hsp25wt) was monomeric with an apparent molecular mass of 25 kDa. A smaller fraction (<20%) had an apparent size of ~50 kDa, which represents the dimeric form of the protein. Following cell exposure to H_2O_2, half of the cellular content of Hsp25 was dimeric, indicating that oxidative stress favored the formation of Hsp25wt dimers. Of interest, in control and oxidative stress-treated cells, the C141A mutation abolished the detection of the dimeric form. It can also be seen in Fig. 1A that the level of Hsp25C141A polypeptide (lane 3) is weaker than that of Hsp25wt (lane 2). As similar levels of total cellular proteins were loaded on each well of the gel, this weaker detection could reflect a decreased stability of the mutant polypeptide. An increase in protein loading (see Fig. 1B) still did not allow the detection of the dimeric form of mutant Hsp25, hence suggesting that cysteine-141 is crucial for Hsp25wt's ability to form dimers.

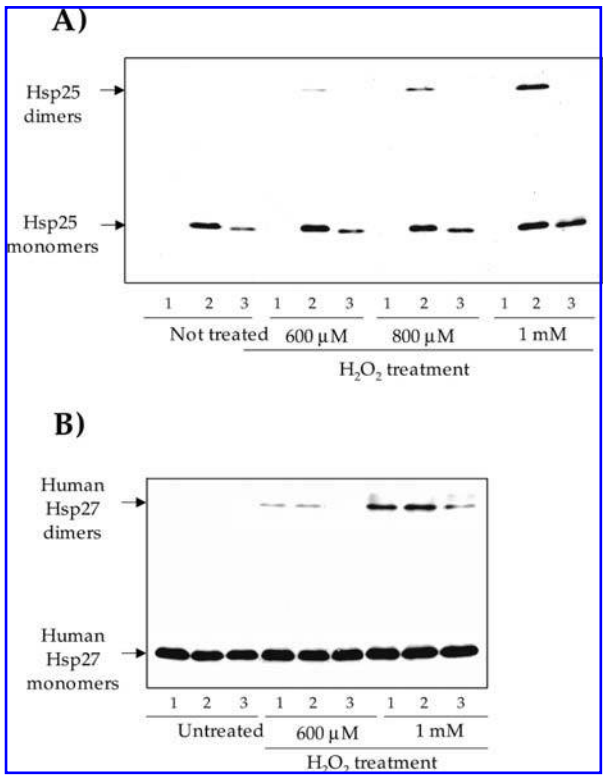

FIG. 2. Characterization of the C141A Hsp25 mutant expressed in human HeLa cells. The experiment was performed in HeLa cells, which constitutively express a high level of human Hsp27. HeLa cells were stably transfected with either the control pCI-neo vector (lanes 1), the pCI-neo-Hsp25wt (lanes 2), or the pCI-neo-Hsp25C141A (lanes 3) vector. Polyclonal populations of transfected HeLa cells were either untreated or treated with various H_2O_2 concentrations to induce exogenous Hsp25 and endogenous Hsp27 dimerization. Immunoblot analysis of exogenous Hsp25 (wt or mutant) **(A)** and endogenous human Hsp27 **(B)** was performed with membranes obtained from SDS-polyacrylamide gels run in nonreducing conditions as described in Materials and Methods. Autoradiographs of ECL-revealed immunoblots are presented.

The same analysis was performed in a polyclonal population of HeLa cells genetically modified to express either Hsp25wt or Hsp25C141A. HeLa cells are characterized by the constitutive expression of human Hsp27 (4). As described above in the case of L929 cells, total cellular proteins were separated in nonreducing gels. Murine Hsp25 and human Hsp27 were then detected in immunoblots probed with specific antibodies. Similar to what was observed in L929 cells, Fig. 2A shows that (a) the formation of Hsp25 dimers is increased by exposing cells to oxidative stress and (b) the C141A mutation abolished the dimerization of Hsp25. When the immunoblots were revealed with human Hsp27 antibody, it is seen in Fig. 2B that human Hsp27, similar to Hsp25, increased its dimerization in cells exposed to oxidative stress. This effect was not affected by Hsp25wt expression. In contrast, the expression of the C141A mutant of Hsp25 (Hsp25C141A) drastically decreased the ability of human Hsp27 to dimerize in response to oxidative stress. This observation strongly suggests that Hsp25C141A interacts with human Hsp27 and abolishes its ability to dimerize. The effect is probably a consequence of the ability of sHsps to interact with each other and form mosaic oligomeric structures (52). Similarly, the coexpression of Hsp25wt and Hsp25C141A polypeptides in L929 cells decreased Hsp25wt's ability to dimerize (data not shown).

As Hsp25 is a phosphoprotein (48) whose level of phosphorylation is drastically increased by oxidative stress (43), we tested whether the C141A mutation altered the isoform composition of this protein. The two-dimensional immunoblots presented in Fig. 3 show that, similar to Hsp25wt, the mutant Hsp25C141A polypeptide was resolved into three isoforms. However, some differences could be detected at the level of the intensity of the different isoforms (which is indicative of their respective cellular concentration). Indeed, in control and H_2O_2-treated cells, the "b" isoform, corresponding to the monophosphorylated form of Hsp25, was more abundant in Hsp25C141A than in Hsp25wt polypeptide.

sHsps are characterized by their ability to form aggregates of heterodispersed native sizes (1, 3, 25, 28, 43, 46). We therefore compared the pattern of oligomerization of Hsp25wt and Hsp25C141A polypeptides expressed in L929 cells to determine whether the C141A mutation influenced the complex assembly properties of this protein. The western blot analysis of the gel filtration profile is presented in Fig. 4A. It shows that Hsp25wt native size is heterodispersed between 30 and 2,000 kDa. Analysis in nonreducing conditions showed that the dimeric form of Hsp25wt is present only in structures with native molecular masses comprised between about 200 and 700 kDa (Fig. 4B). Analysis of Hsp25C141A polypeptide revealed that its native size was also heterogeneous, but did not exceed 600 kDa (Fig. 4C). This suggests that cysteine-141 and the presence of dimers are essential for the formation of structurally organized large oligomers of Hsp25wt.

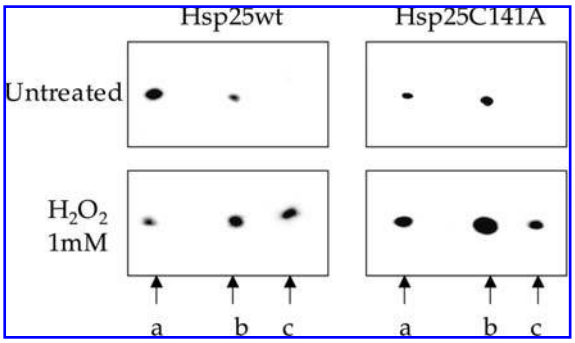

FIG. 3. Two-dimensional immunoblot comparative analysis of the isoform composition of Hsp25 wild type and C141A mutant. L929 cells transiently expressing Hsp25 wild type or C141A mutant were either left untreated or exposed 1 h to 1 m*M* H_2O_2. They were then immediately harvested for two-dimensional immunoblot analysis performed with membranes obtained from SDS-polyacrylamide gels run in reducing conditions as described in Materials and Methods. Autoradiographs of ECL-revealed immunoblots are presented. The acidic end is to the right. Arrows "b" and "c" indicate the mono- and bisphosphorylated isoforms of Hsp25, whereas "a" points to the non-phosphorylated isoform of the protein.

Analysis of the effect mediated by the C141A mutation on the protective activity of murine Hsp25 against oxidative stress

In L929 cells, the expression of human Hsp27, human αB-crystallin, *Drosophila* Hsp27 (3, 26, 28, 41, 44, 49, 51), and murine Hsp25 (40, 43, 44) enhances the cellular resistance to oxidative stress. We therefore tested if the C141A mutation of Hsp25 altered this property. As described above, L929 cells were transiently transfected with pCI-neo, pCI-neo-Hsp25wt, or pCI-neo-Hsp25C141A vector. In addition, to test if the C141A substitution was dominant-negative toward the wild-type protein, cells were transfected with a DNA mixture containing both the pCI-neo-Hsp25wt and pCI-neo-Hsp25C141A vectors. Twenty-four hours after transfection, cells were seeded in six- or 96-well plates for 24 h before being exposed or not to H_2O_2. Figure 5A shows that, following a 1-h exposure of L929 cells to intense oxidative stresses (4 or 8 m*M* H_2O_2), the intracellular production of ROS was strongly decreased by Hsp25wt expression. The protection generated by the C141A mutant was slightly less intense, whereas the concomitant expression of both the wild-type and mutated Hsp25 strongly inhibited this protection and even stimulated the production of ROS. Cell survival analysis, presented in Fig. 5B, reveals that Hsp25wt expression generated a significant protection against a 24-h exposure to 500 μ*M* H_2O_2 that was not affected by the C141A mutation. In contrast, an intense alteration of the protection was observed in the case of the concomitant expression of the C141A mutant and the wild-type protein. These results may be related to the fact that Hsp25 is oligomeric (43) and can form hybrid oligomers with other sHsps (52). Hence, the C141A mutant is dominant-negative probably through its ability to form hybrid structures with Hsp25wt that are not efficient to protect the cell against oxidative stress.

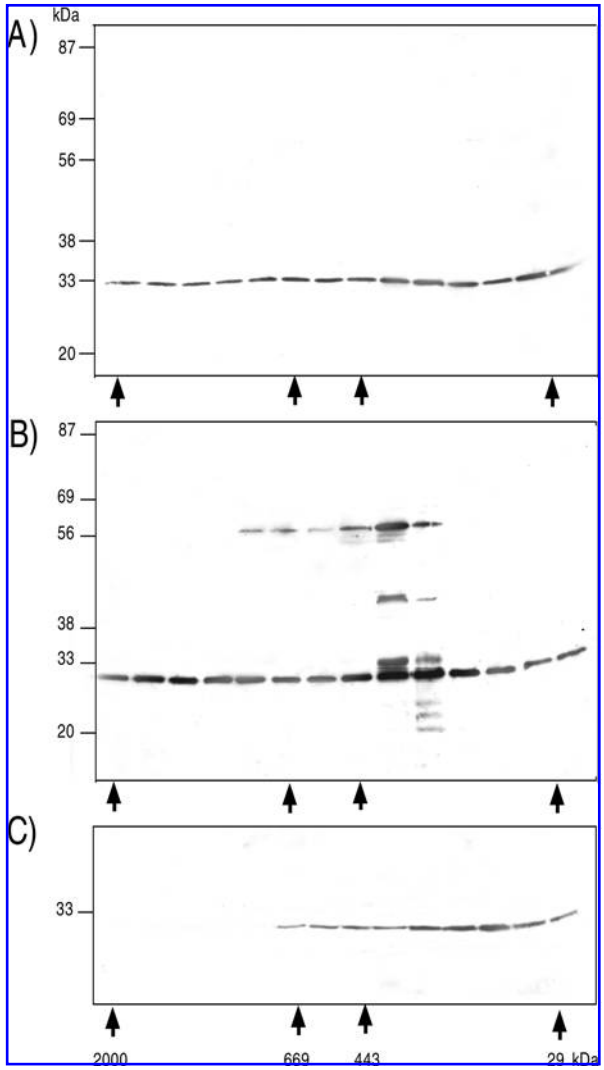

FIG. 4. Analysis of the native size of Hsp25 wild type and C141A mutant. L929 cells stably expressing Hsp25 wild type (WT6 cells) (**A** and **B**) or L929 cells transiently expressing the C141A mutant **(C)** were lysed, and the 20,000 *g* supernatant, which contained the total cellular content of Hsp25, was applied to a Sepharose 6B gel filtration column as described in Materials and Methods. The presence of Hsp25 wild type and the corresponding C141A mutant in the fractions eluted from the column was detected in immunoblots probed with anti-Hsp25 antibody. The arrows 29, 443, 669, and 2,000 indicate the apparent size of gel filtration markers. Immunoblot analysis were performed with membranes obtained from SDS-polyacrylamide gels run in either reducing (A) or nonreducing (B and C) conditions.

Analysis of the effect mediated by the C141A mutation on the protective activity of murine Hsp27 against staurosporine-induced apoptosis

Staurosporine is a kinase inhibitor that induces programmed cell death through an apoptotic pathway whose induction is oxidative stress-independent (19). In L929 cells, the expres-

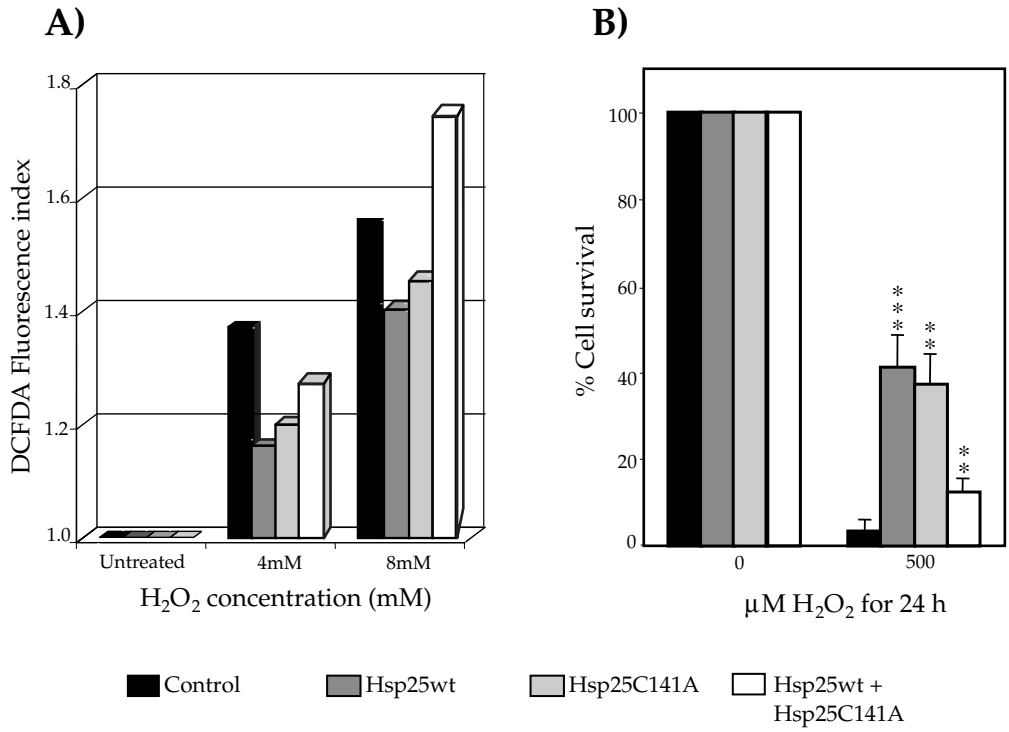

FIG. 5. Effect of the C141A mutation on Hsp25-mediated cellular resistance to oxidative stress. (A) ROS analysis. L929 cells were transiently transfected with either the control pCI-neo vector (black columns), pCI-neo-Hsp25wt vector (dark gray columns), pCI-neo-Hsp25C141A vector (light gray columns), or DNA made of both the pCI-neo-Hsp25wt and pCI-neo-Hsp25C141A vectors (white columns). Forty-eight hours after transfection, cells were either left untreated or treated for 1 h with 4 or 8 m*M* H_2O_2. Cells were then washed with PBS and incubated with DCFDA, and ROS levels were determined by FACS cytometry as described in Materials and Methods. Results are presented as fluorescence indexes calculated by the ratio dividing the fluorescence of treated cells by that measured in the corresponding untreated cells. A representative experiment is presented. **(B)** Survival of L929 cells transfected as above in A. Cells were allowed to grow for 24 h in 96-well plates. They were then either left untreated or treated for 24 h with 500 µ*M* H_2O_2. The remaining viable cells attached to the bottom of the well were stained with crystal violet, and the absorbance of each well was determined. Results are presented as percentage of survival based on the ratio of the absorbance of treated cells to that measured in the corresponding untreated cells. Standard deviations are indicated. The asterisks denote statistical significance when compared with respective controls (**$p < 0.005$; ***$p < 0.001$).

sion of several sHsps, including human Hsp27, was found to delay the apoptotic death induced by this agent (31, 41, 42). To analyze the effects induced by the expression of murine Hsp25 and of its C141A mutation, L929 cells were transiently transfected as described above in the case of oxidative stress. Analysis of cell survival revealed that Hsp25wt generated a protection against staurosporine-mediated apoptosis (Fig. 6). In these conditions, the expression of the C141A mutant was less efficient than that of the wild-type protein. Moreover, as observed in the case of oxidative stress, the C141A mutant/wild-type concomitant expression abolished this protection. The wild-type/C141A structure appears therefore unable to protect against an apoptotic cell death induced by staurosporine.

Analysis of the effect mediated by the C141A mutation on the in vivo *chaperone activity of murine Hsp27*

Decreased luciferase activity and solubility were found in lysates from heat-shocked mammalian cells (35). These characteristics were taken as an indication of thermal denaturation *in situ*. The heat-inactivated luciferase was partially reactivated during recovery after stress. It is also known that the different Hsps, including human Hsp27, act *in vivo* as molecular chaperones favoring the recovery of this activity (36). We therefore analyzed whether the C141A mutation in Hsp25 altered this activity. This was assessed by transiently transfecting L929 cells with DNA mixtures containing the luciferase-expressing vector pGL3 in the presence of either the pCI-neo void vector or the vectors expressing either the wild type or C141A mutant of Hsp25. Forty-eight hours after transfection, cells were exposed or not to 42°C heat shock treatments and allowed to recover at 37°C for different time periods. Luciferase activity was then determined in cell extracts as described in Materials and Methods. We observed that *in vivo* luciferase activity was highly heat-sensitive and drastically dropped after a short exposure of the cells to heat shock. In our cell system, >95% of the activity was lost after 15–20 min of heat shock treatment. After 60 min of recovery, the luciferase activity was slowly regenerating, but it still represented only 6% of the value observed in untreated cells (Fig. 7). This figure also shows that the expression of Hsp25wt interfered with the heat-induced luciferase inactivation. In the presence of Hsp25wt, luciferase activity was two to three times less inac-

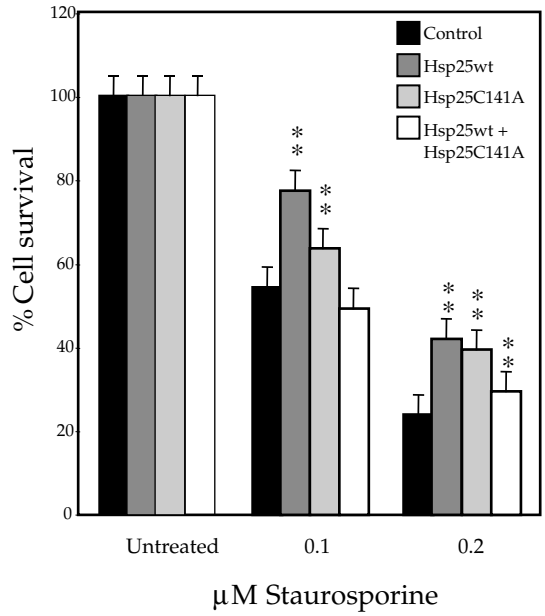

FIG. 6. Effects of the C141A mutation on Hsp25-mediated cellular resistance to staurosporine. L929 cells were transiently transfected as described in the legend of Fig. 5. They were then either left untreated or exposed 24 h to either 0.1 or 0.2 m*M* staurosporine. The remaining viable cells were stained with crystal violet, and the absorbance of each well was determined. Results are presented as percentage of survival based on the ratio of the absorbance of treated cells to that measured in the corresponding untreated cells. Control (black columns), Hsp25wt (dark gray columns), Hsp25C141A (light gray columns), Hsp25wt + Hsp25C141A (white columns). Standard deviations are indicated. The asterisks denote statistical significance when compared with respective controls (**$p < 0.005$).

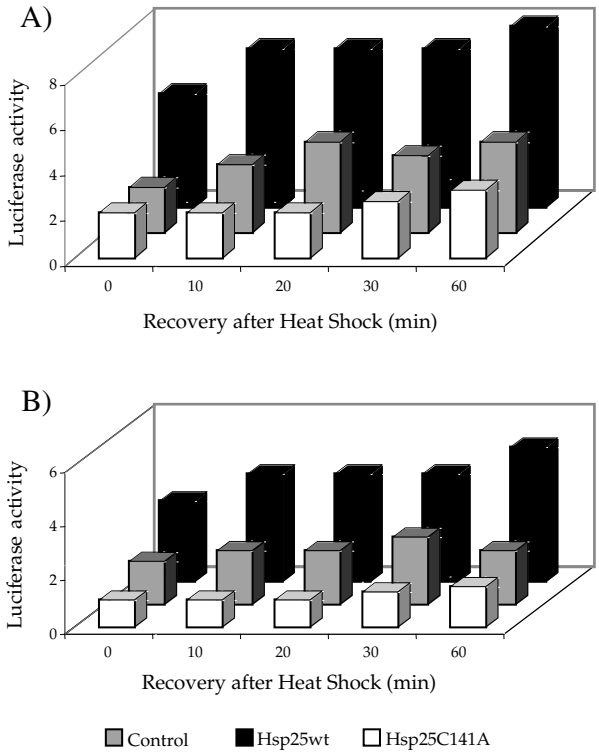

FIG. 7. Effects of the C141A mutation on the *in vivo* chaperone activity of Hsp25. L929 cells transiently expressing luciferase and either Hsp25wt (black columns), Hsp25C141A (white columns), or no Hsp25 polypeptide (gray columns) were scraped and resuspended in fresh medium. Heat shock was performed at 42°C for either 15 **(A)** or 20 **(B)** min, and cells were either immediately analyzed or allowed to recover at 37°C for different time periods up to 60 min before luciferase activity determination (see Materials and Methods). Results are presented as percentage of luciferase activity based on the ratio of the activity measured in treated cells to that of corresponding nontreated cells. Note the similar behaviors of Hsp25wt and Hsp25C141A mutant following 15 min or 20 min of heat shock treatment.

tivated than in control cells transfected with the empty vector. This apparently weak effect of Hsp25wt has to be relativized because in these experiments only ~30% of the cells were efficiently transfected. Analysis of the first 60 min after the heat shock treatment revealed that Hsp25 expression did not significantly alter the kinetics of recovery of luciferase activity compared with those observed in mock-transfected cells. Expression of the C141A mutation of Hsp25 did not interfere with luciferase inactivation, but abolished the recovery of luciferase activity after heat shock. Hence, the C141A mutation efficiently abolishes the *in vivo* chaperone activity of Hsp25 toward heat-induced damaged proteins. This lack of chaperone activity was observed in an L929 cell context devoid of endogenous Hsp25wt expression. This suggests that the C141A mutant lack of chaperone function does not need the formation of dominant-negative mosaic oligomers with the corresponding wild-type protein to be active.

DISCUSSION

Mammalian Hsp27 polypeptides have the capacity to dimerize particularly when cells are exposed to oxidative stress conditions. The results presented here demonstrate that the unique cysteine residue (cysteine-141) of murine Hsp25 is crucial for the dimerization potential of this protein. As shown in our study, by substituting cysteine-141 with an alanine residue, the resulting C141A mutant of Hsp25, analyzed in nonreducing SDS-polyacrylamide gel conditions, did not form dimers when it was expressed in either murine L929 or human HeLa cells. The lack of dimer formation was still observed in cells exposed to oxidative stress. We also observed that in HeLa cells, the C141A mutant, but not the corresponding wild-type polypeptide, interfered with the oxidative stress-mediated formation of dimers by human Hsp27, a constitutively expressed sHsp in these cells. sHsps of different origins can interact with each other, and this interaction does not rely only on disulfide bond formation (52). Hence, in HeLa cells, the C141A nonreactive Hsp25 polypeptide is probably complexed with human Hsp27. This may mask the availability of the unique cysteine residue (C137) of Hsp27 and affect the function of this Hsp. The C141A mutation did not change much the isoform composition of Hsp25 except for the monophosphorylated form, which was more abundant in the mutant compared with the wild-type polypeptide. This may suggest slight structural modifications

that affect the recognition of these polypeptides by MAPKAPK2/3 kinase (48) or the corresponding phosphatase (13).

Hsp25 is an oligomeric protein that shows heterodispersed native size (3, 43, 46) ranging between 30 and 2,000 kDa. Analysis of the native size distribution of the monomeric and dimeric forms revealed that monomers ranged from 30 to 2,000 kDa, whereas, intriguingly, dimers displayed a more restrained distribution comprised roughly between 200 and 700 kDa. This observation suggests that, in the cell, dimers only exist in Hsp25 structures with native molecular masses comprised between 200 kDa and 700 kDa. The significance of this observation is unknown. Of interest, the Hsp25C141A mutant polypeptide was still able to multimerize, but its native size was smaller and ranged only between 30 and 600 kDa. This suggests that cysteine-141 is an essential residue for the *in vivo* formation of Hsp25wt oligomers larger than 600 kDa. Taken together, our data suggest that the *in vivo* structural organization of Hsp25 is complex and does not rely only on dimers and/or tetramers as building blocks of large oligomers. In support of this hypothesis, it has been reported that (a) *in vitro,* the structural organization of recombinant Hsp25 prepared as an oxidized dimeric or reduced monomeric appeared identical (53) and (b) the multimerization of Chinese hamster Hsp27 requires phosphorylation-sensitive interactions at the amino-terminus (21).

Expression of the C141A mutant still generated protection against oxidative stress, but was less efficient toward staurosporine-induced apoptosis and was unable to display *in vivo* chaperone activity. This lack of protection may be related to the fact that tetramers assembled from dimers play a role in the generation of Hsp25 large oligomers that are active *in vivo* after heat treatment (11). Our data also suggest that the modulation of the intracellular redox state and protection against H_2O_2-induced cytotoxicity may not require the C141-dependent chaperone activity. Whether Hsp25C141A mutant, similar to Hsp25wt, is still able to modulate the activity of G6PDH (44) will merit further investigations.

Of particular interest is the dominant-negative effect exerted by the C141A mutant over wild-type Hsp25. This is consistent with a negative dominance through a *trans*-conformational effect resulting in the formation of hybrid oligomers. Other experiments suggest that a similar phenomenon also occurs in the case of human Hsp27. For example, we have observed that the substitution of the unique cysteine residue of human Hsp27 (C137A mutant) induces a negative dominance toward the cellular protection mediated by human Hsp27 against oxidative stress (Arrigo AP, Virot S, Chaufour S, Firdaus W, Kretz-Remy C, and Diaz-Latoud, *Antioxid Redox Signal* 7: 414–424, 2005) or etoposide-induced cell death (7). Hence, as human Hsp27 belongs to the "survival protein family" (18) that participates in the maintenance of aggressively growing and therapy-resistant tumors, the kind of dominant-negative mutant described here may have some future potential therapeutic use.

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ABBREVIATIONS

Carboxy-H2-DCFDA, carboxy-2′,7′-dichlorodihydrofluorescein diacetate; G6PDH, glucose-6-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; Hsp, heat shock protein; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; sHsp, small heat shock protein.

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